Quantitative determination of lappaconitine in plasma by liquid chromatography-tandem mass spectrometry and its application in the pharmacokinetic study in rabbits

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A high sensitive and rapid method was developed for the analysis of lappaconitine in rabbit plasma using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Blood was taken from the ear vein after transdermal administration of lappaconitine gel on the back of male rabbits, followed by liquid-liquid extraction with n-hexane from plasma, the analyte was separated in an Inertsil ODS-3 column (2.1×50mm, 0.5μm) through gradient elution with acetonitrile-water-formic acid at a flow rate of 0.4 mL/min. Detection was performed by positive ion electrospray ionization (ESI) in multiple reaction monitoring (MRM) mode, monitoring the transitions m/z 585.5 → m/z 535.5 and m/z 531.3 → m/z 489.4, for the quantification of lappaconitine and the internal standard (IS) ketonconazole, respectively. The method was linear over the concentration range of 13.125-1050.0 ng/mL and the lower limit of quantification was 13.125 ng/mL. The intra- and inter-day precisions were less than 7.51%, and accuracy ranged from 92.3 to 102%. Under the transdermal concentrations of 13.125 ng/mL, 65.625 ng/mL and 525.00 ng/mL, the absolute recoveries of lappaconitine were 77.8, 84.4 and 79.0%, respectively. The concentration versus time curve in 24 h was fitted using the bimodal model of DAS ver1.0 software and the results showed that the elimination process of lappaconitine in the body of rabbit was similar to the one-compartment model.

Key words: Lappaconitine, LC-MS/MS, plasma drug concentration, pharmacokinetics.

INTRODUCTION

Lappaconitine, a diterpenoid alkaloid extracted from the roots of Aconitum Sinomontanum Nakais (Chen et al., 1980; Wang et al., 1992; Ma et al., 1998), has strong central analgesic, local anesthesia, antifebric and anti-inflammatory activities so as to be the first developed non-narcotic analgesics in China (Gao and Qi, 2008). Its clinical application is primarily to treat cancer pain, postoperative analgesia, induced abortion and cesarean section analgesia, neuralgia caused by herpes zoster, osteoarthritis of the knee and urinary tract syndrome and so on (Tang and Mo, 2007). Meanwhile, injection therapy is regularly used, especially epidural injection (Lu, 2012). However, lappaconitine has narrow safe range because of its certain toxicity (Li et al., 2012a), and its existing delivery methods of lappaconitine still have many drawbacks, which hinder the further application of the drug.

There are few studies of the pharmacokinetic proper-
ties of lappaconitine in animals or human body. The plasma concentrations of lappaconitine hydrobromide and its similar alkaloids, such as aconitine, mesaconitine and hyaconitine, were determined by high performance liquid chromatography (HPLC) (Guan et al., 2006; Shi et al., 2008), LC-ultra violet (UV) (Yang et al., 1989; Wang et al., 1987; Li, 2010), LC-MS (Hikoto O et al., 1997; Wang et al., 2011) or LC-MS/MS (Sun et al., 1999; Zhang et al., 2008) methods. Because of the low sensitivity and poor specificity of UV detection, it only can be used to determine the plasma concentration of moderate level and the results have large errors and low credibility, which are in contrast with the high sensitivity and strong specificity of MS detection.

In addition, investigation indicates that lappaconitine provides rather appropriate oil-water partition coefficient and percutaneous permeability displayed by its chemical structure. Hence, it is fairly an ideal administration method to be made transdermal absorption preparation (Li et al., 2012a). There are reports of patch, micro-emulsion and gel of lappaconitine at present (Han et al., 2008; Li et al., 2012b; Wang et al., 2009), and their analgesic effects are quite evident (Zhou et al., 2005; Qiu et al., 2012). Based on the above reasons, to further improve the drug delivery and enhance the efficacy, we prepared lappaconitine gel and first developed a rapid and sensitive method to determine the concentration of lappaconitine monomer in rabbit plasma with utilizing liquid chromatography-tandem mass spectrometry (LC-MS/MS) in this study. Determination of drug concentration in plasma can not accurately reflect the efficiency of the transdermal delivery method, but provide scientific basis for optimizing dosage regimen and clinical medication.

MATERIALS AND METHODS

Apparatus, drugs and chemicals

HP1100 Series liquid chromatography system (Agilent, USA); API4000 mass spectrometer (Applied Biosystems, USA); SZ-1 Fast vortex mixer (Jincheng Guosheng Test Instrument Factory, Jintan City, Jiangsu Province); centrifuge (model LD-Z4, Beijing Medical Centrifuge Factory) were used for this study.

Lappaconitine gel was made in our laboratory (Wang et al., 2007). Lappaconitine standard (purity 98.8%) was provided by the Institute of Chinese Materia Medica, China Academy of Traditional Chinese Medicine (TCM). Ketonconazole (purity 99%), as the IS, was from Zhejiang East Asia Medicine Chemical Industry Ltd. Co., N-hexane, acetonitrile, and methanol were all of analytical grade purchased commercially. Double-distilled water was used.

Animals

Male New Zealand rabbits, weighing (2.5±0.5) kg, were provided by the Xing-long Experimental Animal Center, Haidian District, Beijing (certification number SCXK2006-0001). The study adopted a single dose administration of 8.0 mg/kg.

Sample collection

Six male rabbits were fasted 24 h prior to the drug administration but with free access to water. The skin hair on the back at an area of 2×10 (cm2) was shaved. Each rabbit was uniformly spread with 1.0 g lappaconitine gel that was weighed precisely (equivalent to 20 mg lappaconitine, 8.0 mg/kg). The gel was covered with 2×10 (cm2) double-deck gauze for 12 h. The gauze was removed with warm water after 12 h. Three ml blood samples were collected from the vein of the edge of rabbit ear into heparinized tubes before drug administration (0 h) and at 10, 20, 30, 45 min, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 24 h after drug administration. The blood samples were centrifuged at 4000 rpm for 15 min, and the plasma was separated and kept frozen at -20°C until analysis.

LC-MS/MS conditions

Chromatographic separation was performed using C18 Inertsil ODS-3 column (2.1×50 mm, 0.5 μm). The mobile phase A was 2.0 mM ammonium acetate and 0.1% formic acid aqueous solution. Mobile phase B was 0.1% formic acid acetonitrile solution. A gradient elution was carried out at the following schedules: 0 min, 10% B; 2.5 min, 55% B; 3.5 min, 55% B; 4.0 min, 10% B; 7.5 min, 10% B. The flow rate was 0.4 mL/min.

The mass spectrometer parameters were set at: voltage 5.4 kV, collision induced voltage 43 V for lappaconitine and 42 V for ketoconazole, ionization source temperature 650°C, curtain gas pressure 25 psi, sheath gas pressure 50 psi, and auxiliary gas pressure 50 psi. Detection was performed by positive ion electrospray ionization (ESI) in multiple reactions monitoring (MRM) mode. Scan time was 0.2 s.

Sample preparation

Lappaconitine stock solution was prepared by dissolving 1.05 mg lappaconitine standard, which was dried to constant weight in 105°C and weighed precisely, in 50 mL methanol and water (50:50, v/v) to obtain a concentration of 21.0 μg/mL. It was stored in a refrigerator until use. Similarly, 50.00 mg of ketoconazole weighed precisely was dissolved in 50 mL methanol and water (50:50, v/v) to obtain a concentration of 1 mg/mL. Twenty-five microliter (25μL) of ketoconazole solution (1mg/mL) was diluted into 50 mL (500 ng/mL) methanol and water (50:50, v/v) and stored in a refrigerator until use.

The plasma samples were thawed and 1.0 mL plasma was spiked with 100 μL IS solution (500 ng/mL ketoconazole) and 500 μL of 1 mol/L NaOH, and mixed well. Three milliliter (3 mL) of n-hexane was added for extraction. The mixture was vortexed for 2 min and centrifuged for 10 min. The organic phase was collected and evaporated to dryness at 40°C under a nitrogen stream. The residue was reconstituted in 200 μL methanol and water (50:50, v/v). 20μL was injected into the LC-MS system.

Validation of the analytical method

Specificity of the LC-MS/MS method was assessed by analyzing drug-free plasma samples. Rabbit plasma standard samples spiked with various concentrations of lappaconitine were prepared by adding 100 μL lappaconitine standard solution into 900 μL drug free plasma and mixed. The final lappaconitine, concentrations were of 13.125, 26.250, 32.800, 65.625, 262.5, 525.0 and 1050.0 ng/mL. The samples were processed and analyzed to obtain the linear range of the calibration curve. The lowest concentration of the
Figure 1. The full scans mass spectrometry of lappaconitine (A) and IS (B).

RESULTS

Method development

Full scans were performed in the positive ion detection mode to develop the ESI conditions for lappaconitine and IS. Lappaconitine predominantly formed protonated molecular ions [M+H]+ and had the most abundant at m/z 585.5 while IS at m/z 531.3 (Figure 1). Multiple reaction monitoring (MRM) mode with parent/daughter mass transitions of 585.5/535.5 and 531.3/489.4 was used to quantify Lappaconitine and IS, respectively.

Specificity

Drug-free plasma was processed and analyzed by the developed LC-MS/MS method. No interference peaks were observed (Figure 2A). The chromatograms of the plasma spiked with lappaconitine at the concentration of 13.1 ng/mL and IS are shown in Figure 2B. The retention
times of lappaconitine and ketoconazole were 4.13 and 4.67 min, respectively. Chromatograms of the rabbit plasma administered with lappaconitine gel are shown in Figure 2C. The results show that under the chromatographic conditions the peaks of lappaconitine and ketoconazole were well separated with good symmetry, and no endogenous sources of interference were observed, indicating the method is specific for the analysis of lappaconitine.

**Linear range**

Rabbit plasma standard samples spiked with various concentrations of lappaconitine were prepared by adding 100 µL lappaconitine standard solution into 900 µL drug free plasma and mixed. The final lappaconitine concentrations were 13.125, 26.250, 32.800, 65.625, 262.500, 525.000 and 1050.000 ng/mL. The samples were processed and analyzed. The area of lappaconitine to that of ketoconazole (Y axis) were plotted against the concentrations of lappaconitine (X axis). A linear regression analysis with the weighted least square method was performed. The resulting regression equation was $Y=0.209X-1.26$ and the linear regression coefficient $r=0.9959$. The lappaconitine standard curve was linear in the range from 13.125 to 1050.0 ng/mL. Therefore, the lower limit of quantification was determined at 13.125 ng/mL.

**Precision, accuracy, recovery**

Three concentrations of lappaconitine (13.125, 65.625 and 525.00 ng/mL) were prepared and analyzed. Each concentration was analyzed five times in one day or in five consecutive days. Concentration of lappaconitine was calculated using the calibration curves prepared on the same day, and was compared with the nominal concentration to estimate the methodological recovery.
The precisions, accuracies, and recoveries were assessed, which are shown in Table 1. The data indicated that intra-day and inter-day precisions and accuracies were well within the requirement of relevant international practice (China Pharmacopoeia, Part II, 2010).

Limit of detection

A standard plasma sample containing 0.1 ng/mL lappaconitine was prepared and analyzed as described. A signal-to-noise ratio of 5 was obtained, indicating that 0.1 ng/mL lappaconitine was detectable. Therefore, the lower limit of detection of lappaconitine in rabbit plasma was 0.1 ng/mL, which is much lower than the lower limit of quantification.

Matrix effect

Three concentrations of lappaconitine (13.125, 65.625, 525.000 ng/mL) were prepared and each sample was analyzed five times and the peak area was recorded. Meanwhile solutions of lappaconitine standard (26.25, 131.25 and 1050.00 ng/mL) were prepared and directly analyzed. The matrix effects were assessed by comparing the peak area obtained from extracts of spiked plasma samples with the peak area obtained from the direct injection of known amounts of standard solutions of lappaconitine. They are 77.8, 84.4 and 79.0%, respectively, at three concentrations.

Stability

Rabbit plasma samples containing lappaconitine at low, medium and high concentrations (13.125, 65.625 and 525.00 ng/mL) were prepared. The stability of lappaconitine in the plasma, under different conditions, was investigated. Lappaconitine was found to be stable after three freeze-thaw cycles, at 25°C for 24 h, and at -20°C for 60 days. The stability results are summarized in Table 2.

Pharmacokinetic study of lappaconitine gel

The validated method was successfully applied to analyze the plasma concentration of lappaconitine at different times after external administration of lappaconitine gel. The maximum plasma concentration of lappaconitine was 189 ng/mL. The lower limit of quantification of this method was lower than 1/10 of Cmax, which met the requirement of Chinese Pharmacopoeia (version 2010). The averaged plasma concentration-time curve of the lappaconitine gel is shown in Figure 3. The concentration versus time curve was fitted using the bimodal model of DAS ver1.0 software. The results show that the elimination process of lappaconitine in the body of rabbit was similar to the one-compartment model, having a higher r² (0.9994) compared with the two-compartment model (0.685). The calculated pharmacokinetic parameters are shown in Table 3.

DISCUSSION

The reports are less concerning the pharmacokinetic study of lappaconitine and mainly about in vivo metabolic analysis of its hydrobromide and similar alkaloids, which using intravenous injection or oral administration. It is the first study on pharmacokinetics of transdermal delivery of lappaconitine monomer that has never been reported at home and abroad.

Experiment indicated that the elimination process of lappaconitine in the body of rabbit was similar to the one-compartment model after transdermal delivery, which was inconsistent with the literature reported that drug concentration-time data of lappaconitine hydrobromide in mouse plasma were fitted to a two-compartment open model by intravenous injection and oral administration (Guan et al., 2006; Shi et al., 2008). The reasons may be related to the drug form, route of administration and experimental animals. However, the specific reasons deserve further study.

Sample preparation played a key role in the development of this method. Several organic solvents were used to extract lappaconitine from the plasma, which included methanol, acetonitrile, ether, ethyl acetate, isopropyl alcohol, chloroform, dichloromethane, 1,2-dichlorethane, n-hexane, and mixed solvents like n-hexane-dichloromethane-isopropyl alcohol (300:150:15), ether-dichloromethane (2:1) and ethyl acetate-isopropyl alcohol (85:15). The results show chloroform, dichloromethane, 1, 2-dichlorethane and n-hexane-dichloromethane-isopropyl alcohol (300:150:15) extracted the drug efficiently, but they were prone to form emulsions, which led to varied recovery. When more hydrophobic solvent, like n-hexane, was used, the recovery is high and no emulsion was formed. We therefore chose n-hexane as the extraction solvent. We also found that basification of the plasma increased the extraction efficiency. The best result was obtained when the plasma was basified with 1 M NaOH solution.

The transdermal lappaconitine plasma concentration-time curve had shown double peaks, the first time to peak about 0.5 h, the second time about 3 h. There are three possible reasons. For one thing, Lappaconitine is an alkaloid with smaller solubility. It is possible that lappaconitine dissolves in the liquid of the surface of skin, some of them in the medicine are precipitated caused by pH change or solvent decrease. Over time, precipitated crystals re-dissolve and absorb again causing fluctua-
Figure 3. The averaged plasma concentration-time curve after administration of a single dose of lappaconitine gel.

Table 1. Intra-day and inter-day analysis of rabbit plasma samples containing different concentrations of lappaconitine (n=5).

<table>
<thead>
<tr>
<th>Concentration (ng/mL)</th>
<th>Intra-day variation</th>
<th>Methodological recovery (%)</th>
<th>RSD (%)</th>
<th>Inter-day variation</th>
<th>Methodological recovery (%)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( X \pm S )</td>
<td></td>
<td></td>
<td>( X \pm S )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13.125</td>
<td>13.4±0.563</td>
<td>102</td>
<td>5.42</td>
<td>14.3±1.21</td>
<td>109</td>
<td>7.51</td>
</tr>
<tr>
<td>65.625</td>
<td>63.8±2.21</td>
<td>97.2</td>
<td>3.16</td>
<td>63.2±3.37</td>
<td>96.4</td>
<td>5.03</td>
</tr>
<tr>
<td>525.00</td>
<td>484±26.7</td>
<td>92.3</td>
<td>4.53</td>
<td>472±32.2</td>
<td>90.0</td>
<td>5.87</td>
</tr>
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</table>

Table 2. Stability of lappaconitine in the rabbit plasma samples.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Concentration (ng/mL)</th>
<th>RE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low</td>
<td>Medium</td>
</tr>
<tr>
<td>Room temperature, 24 h</td>
<td>12.319</td>
<td>63.433</td>
</tr>
<tr>
<td></td>
<td>12.340</td>
<td>63.545</td>
</tr>
<tr>
<td></td>
<td>12.314</td>
<td>63.597</td>
</tr>
<tr>
<td>Three freeze-thaw cycles</td>
<td>12.612</td>
<td>62.167</td>
</tr>
<tr>
<td></td>
<td>12.579</td>
<td>62.160</td>
</tr>
<tr>
<td></td>
<td>12.609</td>
<td>62.114</td>
</tr>
<tr>
<td>-20°C, 60 day</td>
<td>12.684</td>
<td>63.092</td>
</tr>
<tr>
<td></td>
<td>12.641</td>
<td>62.245</td>
</tr>
<tr>
<td></td>
<td>13.200</td>
<td>62.718</td>
</tr>
</tbody>
</table>

tions of blood concentration.

The difference of crystal size causing different dissolving and absorbing rate results in larger fluctuations of blood concentration. For another, the fat-soluble lappaconitine results in rapid distribution of drug into the tissue after absorption. Second release of drug into the blood appears when it is metabolized. Therefore double peaks appear. Finally, the drug concentration increases because of blood volume in animals reducing caused by large number of blood losing.
CONCLUSIONS

In this paper, the elimination process of lappaconitine in the body of rabbit after transdermal administration of lappaconitine gel was researched. The results indicated the method we established was simple, selective, sensitive and could successfully used for the pharmacokinetic study of lappaconitine gel in rabbits.

To our knowledge, this is the first report on the drug plasma concentrations and pharmacokinetic parameters of lappaconitine monomer in rabbit treated with the therapeutic dose. Our method can be used for the in vivo study of transdermal absorption of other TCM and the results provide information for the development of lappaconitine and its analogs.

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Table 3. The pharmacokinetic parameters of lappaconitine gel (one-compartment model).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>$t_{0.504}$ (h)</th>
<th>$t_{1/2}$ (h)</th>
<th>Time of resorption (h)</th>
<th>CL (L/h/kg)</th>
<th>AUC$_{0-12}$ (ng/mL·h)</th>
<th>AUC$_{0-\infty}$ (ng mL$^{-1}$·h$^{-1}$)</th>
<th>$T_{max}$ (h)</th>
<th>$C_{max}$ (ng/mL)</th>
<th>MRT$_{0}$ (h)</th>
<th>VF (f/h)</th>
<th>Ka (f/h)</th>
<th>Ke (f/h)</th>
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<tbody>
<tr>
<td>$X$</td>
<td>0.231</td>
<td>4.460</td>
<td>2.000</td>
<td>0.014</td>
<td>1208.602</td>
<td>1437.292</td>
<td>0.500</td>
<td>3.000</td>
<td>123.000</td>
<td>189.000</td>
<td>7.900</td>
<td>0.089</td>
</tr>
</tbody>
</table>

CL, Clearance; AUC, area under the curve; VF, ventricular fibrillation; MRT, mean residence time.

REFERENCES